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Citation for published version (APA):

Troost, F. J. J., Saris, W. H. M., Haenen, G. R. M. M., Bast, A., & Brummer, R. J. M. (2003). New method to study oxidative damage and antioxidants in the human small bowel: effects of iron application. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 285(2), G354-G359. <https://doi.org/10.1152/ajpgi.00422.2002>

Document status and date:

Published: 01/01/2003

DOI:

[10.1152/ajpgi.00422.2002](https://doi.org/10.1152/ajpgi.00422.2002)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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AJP - GI 285:354-359, 2003. First published Apr 30, 2003; doi:10.1152/ajpgi.00422.2002

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New method to study oxidative damage and antioxidants in the human small bowel: effects of iron application

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Submitted 26 September 2002; accepted in final form 24 April 2003

Troost, Freddy J., Wim H. M. Saris, Guido R. M. M. Haenen, Aalt Bast, and Robert-Jan M. Brummer. New method to study oxidative damage and antioxidants in the human small bowel: effects of iron application. *Am J Physiol Gastrointest Liver Physiol* 285: G354–G359, 2003. First published April 30, 2003; 10.1152/ajpgi.00422.2002.—Iron may induce oxidative damage to the intestinal mucosa by its catalyzing role in the formation of highly reactive hydroxyl radicals. This study aimed to determine iron-induced oxidative damage provoked by a single clinical dosage of ferrous sulfate and to elucidate the antioxidant defense mechanisms in the human small intestine in vivo. A double-lumen perfusion tube was positioned orogastrically into a 40-cm segment of the proximal small intestine in six healthy volunteers (25 ± 5 yr). The segment was perfused with saline and subsequently with saline containing 80 mg iron as ferrous sulfate at a rate of 10 ml/min. Intestinal fluid samples were collected at 15-min intervals. Thiobarbituric acid reactive substances concentrations as an indicator of lipid peroxidation increased significantly from 0.07 μM (range, 0–0.33 μM) during saline perfusion to 3.35 μM (range, 1.19–7.27 μM) during iron perfusion ($P < 0.05$). Nonprotein antioxidant capacity increased significantly from 474 μM (range, 162–748 μM) to 1,314 μM (range, 674–1,542 μM) ($P < 0.05$). These data show that a single dosage of ferrous sulfate induces oxidative damage and the subsequent release of an antioxidant in the small intestine in vivo in healthy volunteers.

duodenum; epithelium; lipid peroxidation; small intestine; oxidative stress

THE PREVALENCE OF IRON DEFICIENCY anemia in Europe and North America is 1% in adult males and 14% in adult females (5). The usual treatment of iron deficiency anemia involves oral iron supplementation with ferrous salts. Ferrous salts, prescribed in amounts ≤195 mg of elemental iron/day divided over three single dosages as ferrous sulfate, -fumarate or -gluconate, respectively (7), are commonly preferred over ferric salts because of their higher bioavailability. Oral ferrous iron therapy may, however, induce oxidative stress due to its catalyzing role in Fenton chemistry, resulting in the production of highly reactive hydroxyl

radicals (6, 23). They can react with any molecule in their direct environment, resulting in a cascade of reactions in which lipids, proteins, and DNA may get damaged. Production of reactive oxygen species (ROS), such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, is normally balanced by the antioxidant system. Excessive ROS production, however, induces a disturbance in the pro- and antioxidant balance, resulting in oxidative damage.

Oral iron therapy, commonly indicated in people with an elevated risk for developing iron deficiency, such as children, adolescents, the elderly, (pregnant) women, vegetarians, and athletes, may induce considerable gastrointestinal (GI) side effects, such as nausea, vomiting, heartburn, abdominal pain, and constipation, in contrast to intravenous iron treatment (2). Iron-induced oxidative damage in the intestine after oral ingestion of iron supplements may, in part, be responsible for these GI side effects. Additionally, oral iron therapy in patients suffering from GI disorders associated with an impaired mucosal or systemic antioxidant capacity, such as inflammatory bowel disease or celiac disease, may attribute to the development of major oxidative damage (8, 13, 16, 19, 27).

Oral iron supplements were shown to aggravate lactic acid-induced damage to the barrier function of the small intestine epithelium (9) and to promote colon tumorigenesis in mice (22) and inflammatory processes in rats with colitis (1, 20). Moreover, electron paramagnetic resonance spectroscopy in rats in vivo showed that oral iron therapy with ferrous sulfate resulted in iron-mediated oxidative stress through hydroxyl radicals in the small intestine. This stress resulted in a decrease in cell turnover, shortening of microvillus height, and partial or complete erosion of the microvilli in the duodenum (24). Epidemiological studies showed a role of dietary iron in the development of colon cancer (26). Additionally, ROS may induce development of several forms of cancer (4, 18).

Because of the complexity of the direct measurement of oxidative damage in the lumen of the human small intestine in vivo, no data are available regarding the

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effects of clinically prescribed amounts of iron on oxidative damage in the human intestine in vivo. Also, little is known about the antioxidant defense mechanisms against ROS production in the intestine. For this purpose, we developed a perfusion technique to directly quantify oxidative damage and antioxidant capacity in vivo in the small intestine in healthy adults. This study aimed to determine iron-induced oxidative damage provoked by a single clinical dosage of ferrous sulfate in the small intestine in humans and to obtain more information about the antioxidant defense mechanisms in the human small intestine in vivo.

MATERIALS AND METHODS

Subjects

Six healthy nonsmoking volunteers (25 ± 5 yr, 3 males, 3 females) without a history of a GI disorder participated in the present study. Volunteers were in good physical health at the time of participation as checked by medical interview. All subjects gave their written informed consent before participation. The study protocol was approved by the Ethics Committee of the University Hospital Maastricht, Maastricht, The Netherlands.

Protocol

After an overnight fast, a perfusion catheter was ingested orogastrically by each volunteer. The catheter consisted of three silicon tubes connected to a rubber balloon on the distal end of the catheter. One of the tubes called the sample port had three small openings at 5 cm proximal of the distal end of the catheter to enable fluid sampling from the small intestine at the level of the distal end of the catheter. The other two tubes were connected in an open loop with the balloon to enable fluid and air transport in and out of the balloon. A fourth lumen had a single opening at 45 cm from the distal end. This port, referred to as the perfusion port, served to administer a test solution directly into the duodenum.

After installation of the catheter tip into the stomach, the rubber balloon was filled with 1.5 ml of mercury. Subjects were instructed to remain in the supine position on the right hand side with the upper body lifted 45° and the feet raised 20 cm until the balloon entered the duodenum. Positioning of the catheter was checked with continuous pH-registration at the tube tip. When the pH shifted from low (~ 1 – 2.5) to ~ 4 – 6 , the mercury was aspirated and the balloon was subsequently inflated with 15 ml of air. Subjects were instructed to stay in the supine position on the back with the upper body lifted 45° . The catheter propagated subsequently by the peristaltic movements of the GI tract. Catheter positioning was successful when the perfusion port was placed ~ 5 – 10 cm distally from the pylorus. This was checked by a second pH-registration channel, which was located at 5 cm proximal from the perfusion port. After positioning, the balloon was deflated to stop it from progressing in the GI tract. Throughout the experiment, the pH of the second pH channel was continuously registered to ensure proper positioning of the perfusion port. After the perfusion experiment, subjects removed the catheter by pulling it gently.

Via the perfusion port, a saline solution was injected at 10 ml/min during 195 min. This period was necessary to reach steady-state conditions as was observed in pilot experiments (data not shown). Subsequently, a 300-ml saline solution containing 80 mg elemental iron as ferrous sulfate (Merck,

Darmstadt, Germany) was perfused at 10 ml/min during 30 min. Finally, a saline solution was perfused again for 60 min to rinse out the iron solution and intestinal secretions.

Intestinal fluid samples were obtained by using the lever properties of the catheter. After an initial adaptation period of 195 min, intestinal fluid content was levered through the sample lumen into a beaker placed in melting ice. Intestinal fluid excretions were pooled over 15-min intervals and stored at -80°C until analysis.

Biochemical Analysis of Intestinal Fluid Samples

Thiobarbituric acid reactive substances assay. The determination of thiobarbituric acid reactive substances (TBARS) was based on the formation of a colored adduct of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA). A 100 μl sample was added to 900 μl of a reagent [containing 0.12 M TBA, 0.32 M *o*-phosphoric acid, 0.68 mM butylated hydroxytoluene (BHT), and 0.01% (mass/vol) EDTA]. The mixture was incubated for 1 h at 100°C in a water bath. After cooling, the MDA-products were extracted with 500 μl butanol. Then 30 μl of the butanol layer were injected on an HPLC system (Agilent, Palo Alto, CA) equipped with a fluorescence detector, set on an excitation wavelength of 532 nm and an emission wavelength of 553 nm, and a Nucleosil C18 column (150×3.2 mm) particle size 5 μm (Supelco, PA). Samples were eluted with 65% (vol/vol) 25 mM PBS (pH 4.8) and 35% (vol/vol) methanol. A calibration curve was constructed by using malonaldehyde bis(diethylacetal) as standard.

Antioxidant capacity assay. The antioxidant capacity assay was carried out as described previously (24a) with some modifications. The 2,2'-azinobis(3-ethylbenzthiazoline)-sulfonate (ABTS $^{+}$) was produced by incubating a solution of 0.23 mM ABTS and 2.3 mM 2,2'-azobis(2-amidinopropane) hydrochloric acid (ABAP) in 100 mM sodium phosphate buffer pH 7.4 at 70°C until the absorption of the solution at 734 nm was between 0.680 and 0.720. Deproteination was done by mixing the sample with an equal volume of a solution of 10% (wt/vol) TCA. In the reaction of antioxidants with the blue/green ABTS $^{+}$, the blue/green color disappears. This decolorization after 5 min is determined spectrophotometrically at 734 nm. The reduction in absorbance is related to that of Trolox, a synthetic, hydrophilic vitamin E analog, which gives the Trolox equivalent antioxidant capacity (TEAC) value. The TEAC value is calculated as molar Trolox equivalents of the sample.

Glutathione assay. Total glutathione was determined by using the recycling method (25). To 50 μl of a 100 μl sample of a 0.4 mM NADPH/0.3 mM 5,5'-dithiobis(2-nitrobenzoic acid) solution in 143 mM PBS (pH 7.4) containing 6.3 mM EDTA, is added in a 96-well plate. The reaction is then started by adding 50 μl of a solution containing 4 U/ml glutathione reductase. The increase of the absorption ($\Delta A/\text{min}$) is followed for 2 min at 405 nm. The $\Delta A/\text{min}$ of the samples is compared with that of the calibrators and the concentration of total glutathione is calculated.

Uric acid assay. Uric acid was determined in ultra filtrates by using an HPLC method described previously (15) with minor modifications. The sample was filtered over a 3-kDa filter unit (Centrex UF 0.5; Schleicher & Schuell, Dassel, Germany) by centrifugation for 45 min at 14,000 g and 4°C . Then 25 μl of the filtrates was analyzed on the HPLC. The system (Agilent) consisted of a Hypersil BDS C-18 end-capped column (125×4 mm), particle size 5 μm (Agilent), with a mobile phase of 5 mM sodium phosphate buffer (pH 3.3). Detection was performed by using a variable wavelength detector set on a wavelength of 292 nm. Concentra-

tions of uric acid were calculated by using a calibration curve of uric acid.

Statistics

Differences were assessed by univariate ANOVA using SPSS version 10.0 software. Differences were regarded as significant when $P < 0.05$. Multiple comparisons, used to localize time differences, were adjusted with the least significant difference.

RESULTS

One to two hours after ingestion of the catheter, the distal end of the catheter entered the duodenum. Catheter positioning was completed within 5 to 8 h in all volunteers.

TBARS levels were significantly higher at 15, 30, 45, and 60 min after the start of the ferrous sulfate perfusion compared with the baseline level measured at $t = 0$ ($P < 0.05$) and returned to baseline levels after 90 min (Fig. 1).

The TEAC of the intestinal fluid was significantly higher at 30 and 45 min after the start of the ferrous sulfate perfusion compared with the baseline level measured at $t = 0$ ($P < 0.05$) (Fig. 2).

Median alkaline phosphatase concentrations of the perfusate samples did not differ significantly between $t = 0$ and $t = 30$ min (502 ± 377 and 510 ± 363 U/L, respectively).

Uric acid concentrations of the perfusate samples were not significantly different from baseline during the first 75 min after the start of the ferrous sulfate perfusion (Table 1). At $t = 90$, uric acid was statistically lower than baseline values ($P < 0.05$).

Glutathione concentrations in the perfusate samples were under the detection limit of the equipment (<0.10 μ M).

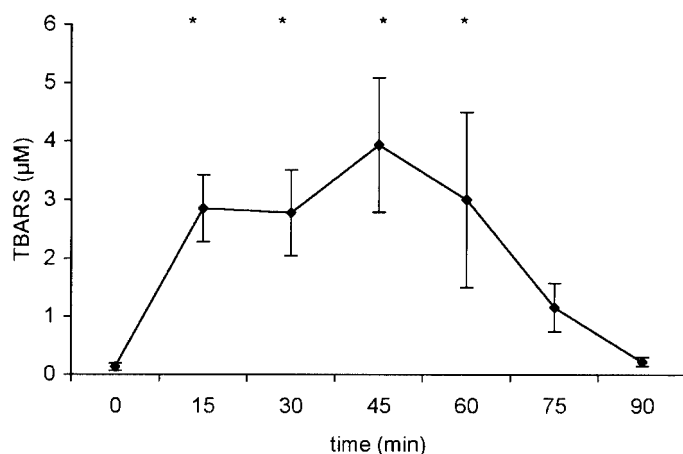


Fig. 1. Thiobarbituric acid reactive substances (TBARS) concentrations in intestinal perfusion samples, collected over 15-min intervals after perfusion of a 40-cm segment in the proximal small intestine in 6 subjects. Baseline values at $t = 0$ were obtained during saline perfusion. Subsequently, saline containing 80 mg elemental iron as ferrous sulfate was perfused for 30 min. Finally, saline was perfused for 60 min at 10 ml/min (means \pm SE; differences vs. $t = 0$ tested with ANOVA; * $P < 0.05$).

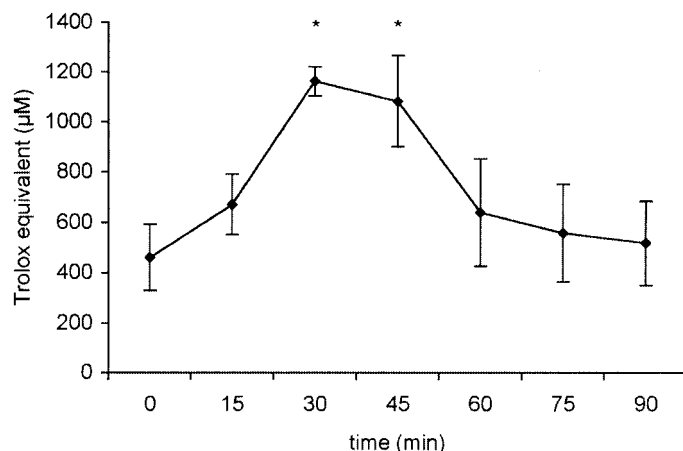


Fig. 2. Antioxidant capacity in intestinal perfusion samples, collected over 15-min intervals after perfusion of a 40-cm segment in the proximal small intestine in 6 subjects. Baseline values at $t = 0$ were obtained during saline perfusion. Subsequently saline containing 80 mg elemental iron as ferrous sulfate was perfused for 30 min. Finally saline was perfused for 60 min at 10 ml/min (means \pm SE; differences vs. $t = 0$ tested with ANOVA; * $P < 0.05$).

DISCUSSION

The present study clearly shows that iron ingestion in vivo induces intestinal oxidative damage as indicated by the occurrence of lipid peroxidation. The magnitude of the TBARS concentration in the intestinal fluid samples as a result of iron ingestion was remarkably high. The TBARS assay was applied to estimate MDA concentration, which is an end product of lipid peroxidation. MDA is a well-accepted parameter of oxidative damage (10). However, it is not specifically produced as a result of free radicals attack, but may be formed during the ex vivo sample incubation stage. To prevent formation of MDA ex vivo, the chain-breaking antioxidant BHT was added. The ability of BHT to prevent TBARS formation during the analytical assay was checked in ex vivo experiments with intestinal fluid samples. In these experiments, we observed an instantaneous rise in TBARS formation on iron addition, but BHT was effective in preventing TBARS formation during the assay. Other possible analytical shortcomings of the conventional TBARS-assay are resolved by the use of the applied HPLC technique (11). Therefore, in the present study TBARS provides an accurate indicator of oxidative damage in vivo. The

Table 1. Uric acid concentrations in intestinal perfusion samples pooled over 15-min periods

Time, min	Uric acid, μ M
0	37.9 ± 15.6
15	34.4 ± 15.7
30	32.5 ± 12.6
45	27.1 ± 8.2
60	25.2 ± 4.8
75	28.7 ± 8.7
90	26.9 ± 10.2

Values are means \pm SD; $n = 6$.



finding that antioxidant capacity increases in the lumen of the small intestine after an oxidative challenge has not been described previously. The rapid and massive increase in antioxidant capacity of the intestinal fluid samples indicates a powerful defense mechanism of the small intestine against exogenous oxidants.

TBARS concentrations in the intestinal fluid samples after the iron challenge are roughly 3–5 times higher than those observed in plasma in healthy adults. This is a highly interesting finding in the view of the dilution of the intestinal fluid samples. In pilot experiments, we measured the dilution of the intestinal fluid with the saline or saline/iron solution in the intestinal segment by the addition of an inert marker, lactulose, to the ingoing solution and subsequent measurement of the lactulose concentration in the perfusate samples. A dilution of ~100-fold was found in all samples. This dilution was constant throughout the experiment (data not shown). In view of the fact that sample dilution did not change during the experiment, independently of the presence or absence of iron, intestinal fluid dilution did not interfere with the results of the study and hence, was not measured in the present study.

On average, ~15 mg of iron is present in the daily diet. Approximately 2.5 mg iron entering the intestine will be absorbed. Approximately 1% of the dietary iron remains in the lumen in a form likely to participate in free radical generation. During oral iron therapy in adults using dosages of 60–195 mg iron/day, this proportion remains the same, resulting in a much larger amount of iron available for Fenton reactions. During Fenton chemistry, ferrous iron catalyzes the formation of hydroxyl radicals in the presence of superoxide anion radicals and hydrogen peroxide, both produced as a result of normal metabolism. Hydroxyl radicals are highly reactive and can inflict damage to any biologic molecule in its direct environment. The free electron can transfer to another molecule and hence, initiate a cascade of damaging reactions. Previously, it was shown that the free radical generating capacity from feces in healthy volunteers was markedly increased after iron supplementation (19 mg/day iron as ferrous sulfate for 14 day) (14). However, this was measured in fecal samples *ex vivo* in an *in vitro* assay. This does not necessarily reflect the conditions from the present study in which the true effects of iron ingestion in the small intestine *in vivo* were investigated. The results of the present study confirm the finding that iron ingestion leads to an increase in free radical production in the gut. The model used in the present study mimics ingestion of an oral iron supplement, which dissolves in the gastric juice and is subsequently released gradually into the small intestine.

From the data (Fig. 1) it is clear that oxidative damage occurs immediately after iron administration into the small intestine. This damage continues during saline perfusion after the ferrous sulfate perfusion. This is likely due to the low perfusion flow in the experimental setup. After discontinuation of the iron perfusion, it takes some time to rinse out all of the iron

and the intestinal juices secreted during the iron perfusion from the small intestine. After 60 min of saline perfusion, no iron-induced rise in TBARS is observed in the intestinal fluid samples anymore. These data are in line with those of a rat study in which it was shown that high dosages of iron supplements induce lipid peroxidation in the colon (21). Any effects of iron supplements in the small intestine were not investigated in that study.

The lipid peroxidation observed in the present study preceded a marked rise in TEAC (Fig. 2). From this, it may be concluded that iron-induced oxidative stress induces the release of an antioxidative component in the small intestine. As for the TBARS concentrations, the TEAC values are also remarkably high, especially in view of the previously mentioned dilution of the intestinal fluid samples with the ingoing solution. The data of the present study indicate that a powerful physiological mechanism in the small intestine protects against iron-induced oxidative damage. In view of the fast release of the antioxidant(s), it is unlikely that the iron-induced oxidative damage directly induced production of an antioxidative agent, which is subsequently released into the lumen. It is more likely that an antioxidant already present in a storage compartment in the small intestine was released on oxidative damage. In the present study, all samples were completely deproteinized during the TEAC assay. Sample deproteination is routine practice during the TEAC assay to reduce the analysis of variance. In pilot experiments, we observed that sample deproteination did not affect the average TEAC values compared with duplicate nondeproteinized samples. Hence, the high antioxidant capacity was exclusively caused by a non-protein species. This excludes a role for enzymatic antioxidants or metallothionines in the observed rise in antioxidant capacity.

The rise in TEAC might be explained by lysis of epithelial or mucosal cells. If the membranes of intact cells were disrupted, its cellular content was excreted into the lumen. As a result, the antioxidant capacity would predominantly originate from the cytosolic antioxidant capacity of the epithelial cells. However, this would have been accompanied by a rise in total alkaline phosphatase, which we did not observe. Also, removal of intact epithelial cells present in the intestinal fluid samples by centrifugation did not significantly affect the antioxidant capacity of the samples (data not shown). This suggests that the strong rise in TEAC after iron-induced oxidative stress was not due to cell lysis but was brought about by a specific antioxidant mechanism.

Data regarding the nonprotein antioxidant capacity of the small intestine are scarce. It was shown in a rat study (17) that the small intestine has a powerful reducing capacity, which is protective against triglyceride hydroperoxides. The underlying mechanism or the site of action (luminal, mucosal, or epithelial) was not identified. In the present study, glutathione and uric acid were determined in the intestinal fluid samples in an attempt to identify the origin of the rise in

antioxidant capacity after iron perfusion. Due to the intestinal fluid dilution by the perfusing solution, glutathione concentrations were below the detection threshold of the equipment used. Uric acid, a powerful antioxidant present in lung- and intestine epithelial lining fluid (12), was present in low amounts not affected by the iron intervention. Vitamin C, present in low amounts in most samples, did not show a correlation with antioxidant capacity (data not shown). Hence, neither of these antioxidants was responsible for the rise in antioxidant capacity after the iron challenge in the present study.

The perfusion model to investigate oxidative stress and antioxidant capacity in the human small intestine enabled us to determine the effects of a prooxidative agent over a standardized segment of the small intestine. A major advantage of the technique is the rather noninvasive protocol of the catheter positioning, because this occurred using normal peristaltic movements. The technique is applicable to healthy volunteers as well as patients. The perfusion technique can only be used to study a limited segment of the proximal small intestine. Hence, results may not be directly extrapolated to the entire small intestine. However, due to a decreasing nutrient concentration along the small intestine, nutrient-associated oxidative damage probably takes place predominantly in the proximal small intestine. The rate of perfusion needs to be as low as possible to avoid effects of the perfusion experiment on normal functioning of the small intestine. Because of the low perfusion flow, it is not possible to monitor acute, short-term responses in the small intestine. Additionally, sampling occurs at 15-min intervals to ensure sufficient sample collection during each collection interval. The perfusion technique provides the unique feature to study oxidative damage and antioxidant capacity in secretions of the small intestine in vivo in humans under strictly standardized conditions. This model is applicable to a variety of intestinal disorders associated with a disturbance in the equilibrium between oxidative stress and the antioxidant defense network. Increased oxidative stress occurs in acute and chronic inflammation, certain drugs, and nutrients. Decreased antioxidant capacity may be present in disorders of mucosal integrity and chronic malabsorption disorders associated with antioxidant deficiencies (8). A disturbance in the equilibrium between oxidative stress and the antioxidant defense network in the small intestine may be an important factor in the pathophysiology of these disorders. Additionally, the perfusion technique can be used to evaluate the efficiency of nutritional antioxidant interventions in restoring a sufficient antioxidant network against oxidative attack in the small intestine.

Iron ingestion induced Fenton-driven oxidative stress in the human small intestine, followed by a massive rise in total antioxidant capacity in the lumen. This rise may reflect the release of a powerful, yet unidentified, nonprotein antioxidant. Future work should focus on identification of this antioxidant and on the possibilities to increase the antioxidant capacity

against oxidative stress with nutritional interventions in disorders related to a high intestinal oxidative stress or a low intestinal antioxidant defense network.

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